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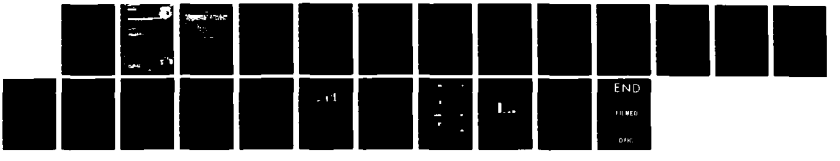

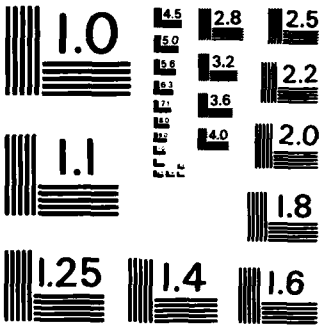


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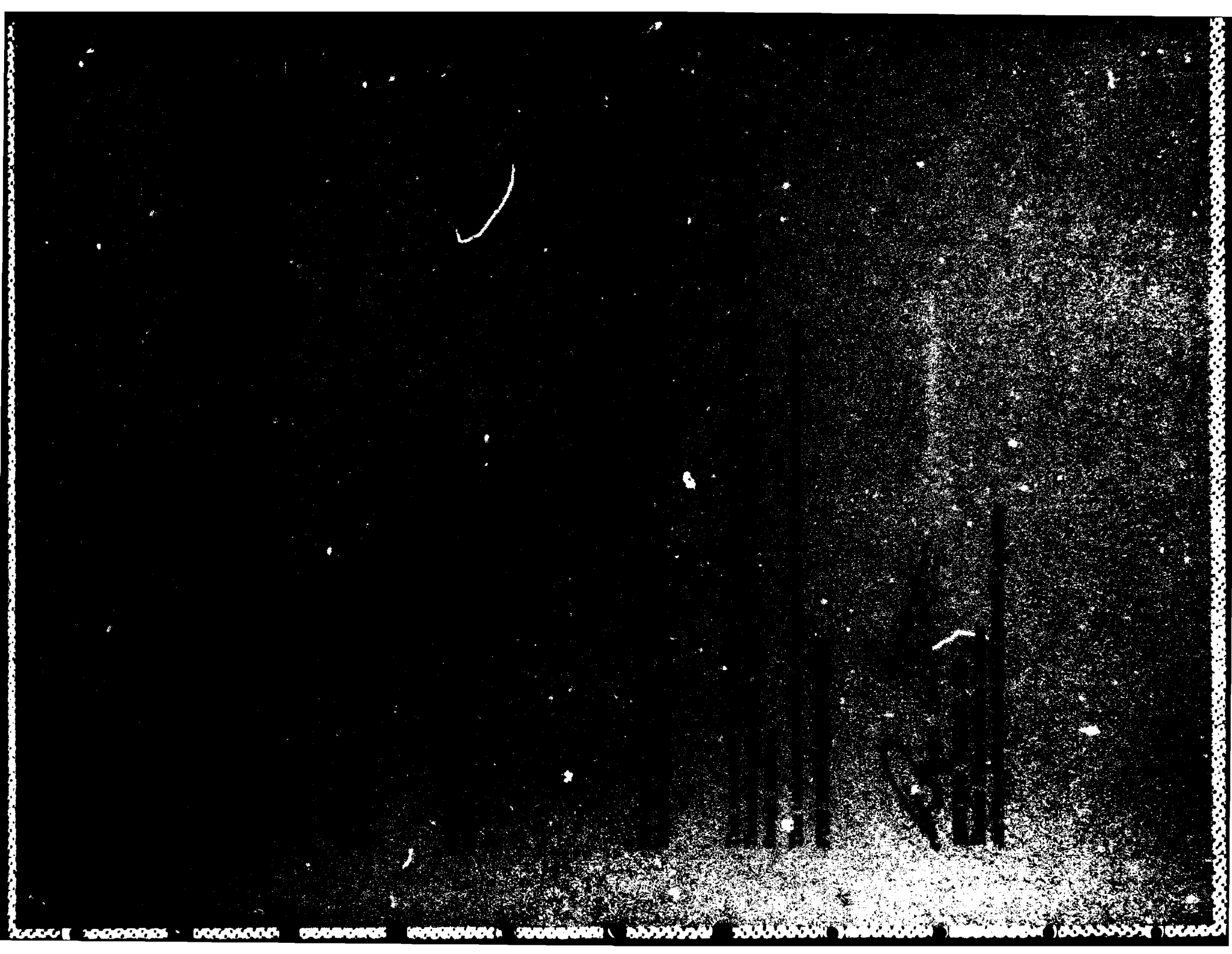

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Pulmonary cells of rats, mice, and hamsters have been separated on the basis of their size by centrifugal elutriation. Primary cultures of cell populations enriched in type II alveolar cells or nonciliated bronchiolar epithelial cells (Clara cells) have been incubated with $^3$H-benzo(a)pyrene ($^3$H-BaP). The conversion of $^3$H-BaP to hydroxylated metabolites and binding of $^3$H-BaP to cellular DNA was greatest in cultures enriched in Clara cells. Pretreatment of the animals with beta-naphthoflavone (BNF) 24 or 48 hr prior to sacrifice increased the metabolic activity toward $^3$H-BaP in both type II and Clara cell enriched cultures. Qualitative and quantitative differences in $^3$H-BaP metabolism were small between species in animals pretreated with BNF, but lung cells from untreated rats showed no detectable metabolism of $^3$H-BaP while Clara cells from untreated mice or hamsters showed considerable activity. The experiments demonstrated that metabolism and binding of this carcinogen could occur entirely within the presumed target cells for its carcinogenic activity. (Cont.)

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Block 19. Abstract, continued.

Formation of $O^6$-ethylguanine ($O^6$-EtG) was studied in hamster respiratory cell DNA after in vivo or in vitro exposure to diethylnitrosamine (DEN). Highest levels of $O^6$-EtG were found in tracheal epithelial cells and in Clara cells isolated from pulmonary tissue by centrifugal elutriation of cell suspensions. DEN is an organotropic carcinogen that produces malignant tumors in the trachea of hamsters. The results indicated that formation of $O^6$-EtG was greatest in those cells that were targets for the carcinogenic action of DEN, and support the hypothesis that this altered DNA base may have a role in the initiation of carcinogenesis.

## PREFACE

This is the fifth annual report of the Cytology, Cell Biology and Cytogenetics section of the Toxic Hazards Research program performed by the Department of Community and Environmental Medicine of the University of California, Irvine (UCI) on behalf of the Air Force under contract number F33615-80-C-0512. The report describes research activities at UCI during the contract period 1 July 1984 through 30 June 1985. During this period, T. T. Crocker, M.D., was Principal Investigator for the contract, and R. E. Rasmussen, Ph.D., directed the studies at UCI. Technical Personnel at UCI were Staff Research Associates Marcia Witte, Mary Hawley, and Gary DeVillez, and Research Assistant Arthur Fong. Technical Monitor for the Air Force was M. K. Pinkerton, AFAMRL/THT, Wright-Patterson AFB, Ohio.

## TABLE OF CONTENTS


PREFACE....1

INTRODUCTION....2

SPECIFIC AIMS....3

MATERIALS AND METHODS....4
- Separation of Lung Cells by Centrifugal Elutriation..4
- Culture of Lung Cells and Measurement of $^{3}$H-BaP Metabolism....5
- Treatment of Hamsters with DEN....6
- Isolation of Hamster Respiratory Cells....6
- Isolation of DNA and Analysis of Ethylated Guanine...6

EXPERIMENTAL RESULTS AND DISCUSSION....7
- Metabolism of $^{3}$H-BaP by Isolated Rat Lung Cells....7
- Metabolism of $^{3}$H-BaP by Mouse Lung Cells....8
- Metabolism of $^{3}$H-BaP by Hamster Lung Cells....9
- Binding of $^{3}$H-BaP to DNA of Lung Cell Cultures....10
- $O^{6}$-EtG Concentrations in Hamster Tracheal DNA After Various Doses of DEN....11
- Persistence of $O^{6}$-EtG in Hamster Tracheal DNA After a Single Large Dose of DEN....13
- $O^{6}$-EtG Formation in Hamster Tracheal DNA After Subchronic DEN Exposure....13
- Accumulation of $O^{6}$-EtG in Hamster Tracheal DNA During Subchronic Exposure to DEN....13
- Cell Specificity in Hamster and Rat Pulmonary DNA Alkylation After DEN or Ethyl Methanesulfonate (EMS) Treatment....13
- $O^{6}$-EtG in DNA of Rat Pulmonary Cells....14
- $O^{6}$-EtG in Pulmonary Cells During Subchronic Exposure to DEN....15
- Metabolism of DEN by Hamster Pulmonary Cells and Hepatocytes in Culture....15

REFERENCES....18

## LIST OF FIGURES


FIGURE — PAGE

1. Formation of $O^6$-EtG in Hamster Tracheal DNA after Various Doses of DEN.....12
2. $O^6$-EtG Content of Hamster Tracheal DNA after a Single Large Dose of DEN.....12
3. $O^6$-EtG content of Hamster Tracheal DNA During Subchronic Exposure to DEN.....15
4. $O^6$-EtG Content of Hamster Lung Cells After In Vivo Exposure to DEN.....16
5. $O^6$-EtG Content of Hamster Cells After In Vitro Exposure to DEN.....17


## LIST OF TABLES


TABLE — PAGE

1. Metabolism of $^3$H-BaP by Rat Lung Cells In Culture.....7
2. Metabolites of $^3$H-BaP Produced by Rat Lung Cells.....8
3. Metabolism of $^3$H-BaP by Mouse Lung Cells in Culture.....9
4. Metabolites of $^3$H-BaP Produced by Mouse Lung Cells.....9
5. Metabolism of $^3$H-BaP by Hamster Lung Cells in Culture....10
6. Metabolites of $^3$H-BaP Produced by Hamster Lung Cells.....10
7. Binding of $^3$H-BaP to DNA in Primary Rat, Mouse and Hamster Lung Cell Cultures.....11


## INTRODUCTION

### Statement of the Problem

The work described in this report has addressed the problem of comparative metabolism of xenobiotics among the common laboratory species of rat, mouse, and hamster. While inbred species usually show a relatively uniform response to xenobiotics in terms of toxicity and carcinogenicity, there are wide interspecies and interstrain differences. Several examples are known of chemicals which are potent carcinogens in one species, but produce little effect in others. These species differences appear to involve humans as well. The working hypothesis for the present studies is that species differences in susceptibility to toxic or carcinogenic effects of chemicals may be related to qualitative and quantitative differences in the metabolism of the chemicals in the cells that are the target for their action.

The problem has been approached in two ways. The first method has been to conduct comparative studies with lung cells isolated from the three common laboratory species with respect to their metabolic activity toward an ubiquitous environmental carcinogen, benzo(a)pyrene (BaP). Specific lung cell types have been separated by centrifugal elutriation and metabolite patterns have been analyzed. The results have indicated that the various lung cell types have widely different levels of enzymatic activity, and that this activity can be modified by prior treatment of the experimental animals. The binding of this carcinogen to cellular DNA, which is thought to be related to its carcinogenic action, is also related to the level of cellular enzymatic activity, and is highest in those cells known to be targets for carcinogens such as BaP. The studies have focussed on two cell types which may be of special importance as targets for carcinogens, the type II alveolar cell and the nonciliated bronchiolar epithelial (Clara) cell. Experimental carcinogenesis studies in rodents as well as morphological studies of human lung tumors have strongly suggested that these cell types are among the targets for carcinogenic chemicals. The results of the studies reported here have confirmed that metabolic activation of BaP and its binding to DNA can occur entirely within the presumed target cells for its carcinogenic action.

The second approach has been to examine xenobiotic metabolism within a single species and to conduct a detailed study of the metabolism and binding of an organotropic carcinogen, diethylnitrosamine (DEN), in target and nontarget cells and tissues of the hamster. In this species DEN induces malignant tumors of the tracheobronchiolar epithelium, regardless of the route of administration. DEN metabolism and binding to DNA has been studied in both target cells and tissues of the respiratory tract, and in nontarget tissues of the liver and kidney. These studies have demonstrated a correlation between binding of DEN to cellular DNA and susceptibility of the particular cell types to neoplastic transformation.

## SPECIFIC AIMS

The program in Cytology, Cell Biology and Cytogenetics is concerned with the precancerous changes which occur in tissues during and following exposure to carcinogens. In past years this program, has focussed on the respiratory system, with the rat and hamster being the principal experimental animals. The work has progressed from experiments involving tumorigenesis dose-response studies, through metabolic and cytogenetic studies with tissue enzymes and cells to the present where ongoing experiments employ isolated lung cell preparations enriched in specific lung cell types. The studies during the present year have concentrated on critical comparative studies among the three common experimental animals (rat, mouse, and hamster).

The program consists of two projects, each aimed at a particular aspect of the mechanism of carcinogenesis at the cellular and molecular levels. One project is concerned with

comparative metabolism of carcinogens by isolated lung cells from the three species. The other project is a detailed study of the metabolism and DNA binding of the organotropic carcinogen DEN in the hamster.

The Specific Aims for the comparative lung cell studies are:

1. To refine methods for isolation of three major lung cell types (type II, Clara, and alveolar macrophage) from the lungs of the three species.

2. To conduct comparative studies with the isolated cells with respect to :

   a. Enzymatic specificity of BaP metabolism;

   b. Response of mixed function oxidase levels to in vivo or in vitro pretreatment with enzyme inducers;

   c. Binding of BaP to cellular DNA.

3. To compare results obtained with the three species with the aim of elucidating major similarities and differences.

The Specific Aims for the study of DEN metabolism and binding in the hamster are:

1. To determine the concentrations of $O^6$-ethylguanine ($O^6$-EtG) and 7-ethylguanine (7-EtG) in hamster tracheal DNA during chronic DEN exposure;

2. To measure the formation and subsequent removal of $O^6$-EtG from tracheal DNA after a large i.p. challenge dose of DEN administered upon cessation of a term of chronic, relatively low level pretreatment;

3. To examine the rate of formation and persistence of $O^6$-EtG and 7-EtG in hamster tracheal DNA after treatment with a single large i.p. injection of DEN;

4. To measure the concentrations of $O^6$-EtG and 7-EtG in hamster tracheal DNA after various single doses of DEN;

5. To measure the concentrations of $O^6$-EtG and 7-EtG in hamster Clara and type II alveolar cells after various single doses of DEN.

## MATERIALS AND METHODS

Separation of Lung Cells by Centrifugal Elutriation.

The preparation of cell suspensions from rodent lung and their separation by centrifugal elutriation has been described in the previous Annual Report (Rasmussen and Fong, 1984). In brief,

the animals were anesthetized with phenobarbital, the lungs lavaged with Krebs-Ringer to remove alveolar macrophages, and then inflated with a mixture of trypsin and hyaluronidase. After removal of the lungs and incubation at 37°C for 20 min, the lobes of the lung were removed, minced, and the freed cells collected by filtration and centrifugation. The cells were resuspended and loaded at a flow rate of 8 mL/min into a Beckman JE-6 elutriator rotor in a J6M centrifuge, rotating at 1500 rpm. The flow rate through the rotor was increased stepwise, and fractions of 150 mL each were collected at each flow rate. The cells eluted from the rotor on the basis of size, with smaller cells eluting first followed by larger cells and cell clumps. With respect to the cells of interest, the type II alveolar cells eluted at a flow rate of 9-11 mL/min and the Clara cells and other bronchiolar epithelial cells at approximately 30 mL/min. These characteristics have been found to be consistent for the three species studied. The final fraction was collected at a flow rate of 32 mL/min after stopping the rotor. This fraction consists largely of cell clumps containing Clara cells and other ciliated bronchiolar epithelial cells, but no type II cells. Attempts to break up the clumps by pipetting or further enzymatic treatment have not been successful. Therefore, this last fraction has been considered to be representative of the bronchiolar epithelial cell population in general.

Culture of Lung Cells and Measurement of $^{3}$H-BaP Metabolism.

Initial studies employed homogenates of isolated cells to examine the conversion of $^{3}$H-BaP to hydroxylated metabolites. In the present series of experiments, the cells isolated by centrifugal elutriation were used to establish primary cultures to which was added $^{3}$H-BaP. This procedure required that sterile precautions be observed throughout the isolation of the cells, including sterile dissection of the animals and sterilization of the centrifuge rotor by flushing with 6% hydrogen peroxide. Although somewhat laborious, these methods have enabled examination of $^{3}$H-BaP metabolism and binding to DNA in intact cells of the three species.

Cultures enriched in either type II alveolar cells or Clara cells were established by seeding 60 mm plastic dishes (Falcon, Oxnard, CA) with 3-4 x $10^{6}$ cells. The culture medium was Dulbecco's minimum essential medium (DMEM) supplemented with 10% fetal bovine serum, 0.1 ug/mL hydrocortisone, 0.2 ug/mL insulin, and 100 ug/mL gentamicin. Purified $^{3}$H-BaP (17-20 Ci/mmole, Amersham Corp., Arlington Hts., IL) was dissolved in dimethyl sulfoxide (DMSO) and added to the culture medium as indicated in the text. The final concentration of DMSO did not exceed 0.1%. After 18-24 hr incubation at 37°C, the medium was removed and frozen. The cells were collected by scraping them from the dishes with a rubber policeman, and frozen at -70°C until isolation of the DNA.

Metabolites of $^{3}$H-BaP present in the medium were analyzed by thin layer chromatography (TLC) as previously described (Rasmussen and Wang, 1974; Rasmussen and Fong, 1984). In brief, aliquots of the culture medium were extracted with ethyl acetate, the organic extracts dried under $N_2$, and chromatographed on silica gel TLC plates using benzene, and benzene:ethanol (9:1) sequentially in the same direction, as solvents. Nonradioactive BaP derivatives for use as TLC standards were obtained from the National Cancer Institute. Radioactivity associated with the metabolites was determined by scintillation spectroscopy.

DNA was isolated from the cells by lysis with sodium dodecyl sulfate (SDS), deproteinization with $CHCl_3$:isoamyl alcohol (24:1), and banding in CsCl as described previously (Rasmussen and Painter, 1966).

Treatment of Hamsters with DEN

Male Syrian golden hamsters were given DEN by ip injection of solutions in 0.9% NaCl as appropriate to the experiments. The hamsters weighed 90.0 $\pm$ 2.5 g at the start of an experiment. Waste DEN was destroyed by alkali treatment before disposal.

Isolation of Hamster Respiratory Cells.

Preparations enriched in either type II or Clara cells were obtained in the same manner as described above. Tracheal epithelial cells were obtained by scraping the epithelium of excised tracheas, collecting the cells by centrifugation, and freezing at -70$^{o}$C until analysis of the DNA.

Isolation of DNA and Analysis of Ethylated Guanine.

DNA was isolated using either a modified phenol extraction procedure (Swann and Magee, 1968) or by lysis of cell suspensions on membrane filters (Leadon and Cerutti, 1982). The latter method offers the advantage that fewer cells are needed for each analysis, and therefore the numbers of cells obtained by centrifugal elutriation or from tracheal epithelium are sufficient to allow determination of alkylated guanines. Once isolated, the DNA was analysed for alkylated guanines by high performance liquid chromatography (HPLC) as previously described (Rasmussen and Fong, 1984). The content of $O^6$-EtG was calculated as micromoles of $O^6$-EtG per mole DNA guanine (umol/mol G).

The Specific Aims included the intent to analyze 7-ethylguanine (7-EtG) as well as $O^6$-EtG content of DNA from treated animals and cells. However, the conditions of HPLC analysis favorable for quantitation of $O^6$-EtG were not suitable for simultaneous quantitation of 7-EtG. During HPLC the 7-EtG peak did not totally resolve from the preceding adenine peak, making accurate quantitation of 7-EtG difficult, especially with the small amounts of DNA obtained from tracheas and isolated pulmonary cells. The merged adenine and 7-EtG peaks combined

with the low fluorescence intensity of 7-EtG relative to $O^6$-EtG made quantitation of 7-EtG impractical. Attempts to remedy the problem by changing solvent systems and gradient patterns were unsuccessful. Therefore, a decision was made to focus on $O^6$-EtG content alone. A further rationale for this decision is that the formation of $O^6$-EtG in DNA has been correlated with tumorigenesis and mutagenesis in a number of experimental systems, while no such association has been shown for 7-alkyl-guanine.

## EXPERIMENTAL RESULTS AND DISCUSSION

### Metabolism of $^3$H-BaP by Isolated Rat Lung Cells.

Rat lung cells isolated and cultured as described were incubated with $^3$H-BaP and the metabolites appearing in the medium analyzed by TLC. In these experiments each culture dish contained 2.5-3 x $10^6$ cells. Prior to isolation of the lung cells the rats were treated with beta-naphthoflavone (BNF, 80 mg/kg b.w.) given by ip injection as a peanut oil solution. The conversion of $^3$H-BaP to water soluble metabolites is shown in Table 1. Cells from untreated rats showed no detectable metabolism of $^3$H-BaP. The metabolism of $^3$H-BaP by cells from BNF-treated rats was related to the time after treatment with the cells from rats killed at 48 hr posttreatment being more active. At 24 hr, the Clara cell-enriched cultures appeared more active than the type II-enriched cultures but, at 48 hr, the cultures were equally active in conversion of $^3$H-BaP to water soluble derivatives. The nature of the water-soluble metabolites was not determined but, based on the work of others (Cohen et al., 1976), they probably consisted of sulfate and glucuronide conjugates. The ethyl acetate extract contained $^3$H-BaP and hydroxylated metabolites of $^3$H-BaP.

TLC analysis of the ethyl acetate extracts is shown in Table 2. The extract of medium from cultures containing cells from untreated rats contained only unchanged $^3$H-BaP. The most abundant single metabolite was the 9,10-diol, which made up 1/3 to 1/2 of the total metabolite yield with either cell type. Radioactivity at the origin of the TLC plates was not further analyzed, but probably consisted of triols and tetrols. At 24 hr, the Clara cell-enriched cultures appeared to be most active, but at 48 hr, the total metabolite yield as well as the relative proportions of each metabolite was similar for both cell types.

### Metabolism of $^3$H-BaP by Mouse Lung Cells.

Conversion of $^3$H-BaP to water-soluble metabolites by isolated mouse lung cells is shown in Table 3. With cells from untreated mice, the cultures enriched in type II cells showed little activity. However, in contrast to the rat, the Clara cell cultures were quite active in the production of water-soluble derivatives. At 24 and 48 hr after treatment with BNF both cell types showed increased activity over the controls, but the Clara cell-enriched cultures were clearly more active at both times.

**TABLE 1**

Metabolism of $^{3}$H-BaP by Rat Lung Cells

Values are the percent of radioactivity extracted from the culture medium into ethyl acetate and the percent remaining in the aqueous phase. Recovery values are the total of ethyl acetate and aqueous phase radioactivity divided by the amount of $^{3}$H-BaP originally added to the culture medium times 100.

| TREATMENT | CELL TYPE | ETOAC | AQUEOUS | RECOVERY |
|---|---|---|---|---|
| None | Type II | 93.9 | 6.1 | 62 |
| None | Clara | 92.9 | 7.1 | 59 |
| BNF, 24 hr | Type II | 78 | 22 | 58 |
| BNF, 24 hr | Clara | 45 | 55 | 67 |
| BNF, 48 hr | Type II | 24 | 76 | 72 |
| BNF, 48 hr | Clara | 28 | 72 | 68 |
| BNF, 48 hr | Type II | 35 | 65 | 70 |
| BNF, 48 hr | Clara | 31 | 69 | 68 |
| Blank | No Cells | 94 | 6.0 | 64 |

**TABLE 2**

Metabolites of $^{3}$H-BaP Produced by Rat Lung Cells

The values are the percent of the ethyl acetate extractable radioactivity appearing as the individual metabolite on TLC. Values for BaP are the percent of the ethyl acetate extract appearing as unchanged $^{3}$H-BaP. No metabolites were detected with cells from untreated rats.

| | BNF, 24 hr Before Sac. | | BNF, 48 hr Before Sac. | |
|---|---|---|---|---|
| Metabolite | Type II | Clara | Type II | Clara |
| Origin | 10.7 | 11.3 | 17.7 | 19.8 |
| 9,10-Diol | 30.0 | 52.1 | 43.5 | 45.0 |
| 7,8-Diol | 12.3 | 11.1 | 11.3 | 7.6 |
| 4,5-Diol | 6.4 | 4.5 | 4.8 | 3.9 |
| Unknown | 4.8 | 4.1 | 3.8 | 5.1 |
| Monohydroxys | 12.7 | 6.9 | 8.6 | 7.8 |
| Quinones | 23.1 | 10.1 | 9.4 | 10.6 |
| BaP | 97.3 | 90.5 | 85.7 | 88.1 |

Analysis of the metabolites of $^{3}$H-BaP in the ethyl acetate extracts is shown in Table 4. As in the case of the rat cells, the major metabolite was the 9,10-diol, but the 7,8-diol was also a prominent metabolite with both cell types. The Clara cell-

enriched cultures were also more active in production of metabolites remaining at the origin of the TLC plates.

**TABLE 3**

Metabolism of $^{3}$H-BaP by Mouse Lung Cells

Values are as in Table 1.

| TREATMENT | CELL TYPE | ETOAC | AQUEOUS | RECOVERY |
|---|---|---|---|---|
| Untreated | Type II | 95.0 | 5.0 | 61 |
| Untreated | Clara | 80.5 | 19.5 | 67 |
| BNF, 24 hr | Type II | 84.3 | 15.7 | 75.2 |
| BNF, 24 hr | Clara | 31.3 | 68.7 | 66.2 |
| BNF, 48 hr | Type II | 88.8 | 11.2 | 60 |
| BNF, 48 hr | Clara | 40.2 | 59.8 | 74 |
| Blank | No Cells | 96.1 | 3.9 | 81.7 |

**TABLE 4**

Metabolites of $^{3}$H-BaP Produced by Mouse Lung Cells

Values are as in Table 2.

| | UNTREATED MICE | | BNF 24 HR PRIOR | | BNF 48 HR PRIOR | |
|---|---|---|---|---|---|---|
| METABOLITE | TYPE II | CLARA | TYPE II | CLARA | TYPE II | CLARA |
| Origin | N.D.* | 9.8 | 11.2 | 50.0 | 11.6 | 16.0 |
| 9,10-Diol | " | 31.8 | 35.0 | 9.8 | 36.0 | 53.1 |
| 7.8-Diol | " | 35.2 | 29.1 | 11.3 | 28.0 | 18.5 |
| 4,5-Diol | " | 4.3 | 7.3 | 7.0 | 4.2 | 2.7 |
| Unknown | " | 2.5 | 2.9 | 5.5 | 3.7 | 2.5 |
| Monohydroxys | " | 14.2 | 10.9 | 7.9 | 7.9 | 4.3 |
| Quinones | " | 2.2 | 3.6 | 12.5 | 8.5 | 2.8 |
| BaP | 98.45 | 92.06 | 92.16 | 64.9 | 95.4 | 76.7 |

*N.D. = Not Detected.

Metabolism of $^{3}$H-BaP by Hamster Lung Cells.

The conversion of $^{3}$H-BaP to water soluble metabolites by hamster lung cell cultures is shown in Table 5. Cultures enriched in Clara cells from both untreated and BNF-pretreated hamsters showed significant activity; however, the cultures enriched in type II cells showed activity only in the cultures prepared from hamsters pretreated 48 hr previously, and then only showed about half the activity of the Clara cell cultures.

**TABLE 5**

Metabolism of $^{3}$H-BaP by Hamster Lung Cells

Values are as in Table 1.

| TREATMENT | CELL TYPE | ETOAC | AQUEOUS | RECOVERY |
|---|---|---|---|---|
| Untreated | Type II | 94.5 | 5.5 | 82.9 |
| Untreated | Clara | 79.6 | 20.4 | 85.7 |
| BNF, 24 hr | Type II | 93.2 | 6.8 | 96.9 |
| BNF, 24 hr | Clara | 77.9 | 22.1 | 94.8 |
| BNF, 48 hr | Type II | 81.0 | 19.0 | 75 |
| BNF, 48 hr | Clara | 52.0 | 48.0 | 82 |

Analysis of $^{3}$H-BaP metabolites in the ethyl acetate extracts showed that, as with the rat and mouse lung cells, the major metabolite was the 9,10-diol (Table 6). A smaller fraction of the total metabolites remained at the origin of the TLC plates, suggesting a lower production of triols and tetrols.

**TABLE 6**

Metabolites of $^{3}$H-BaP Produced by Hamster Lung Cells

Values are as in Table 2.

| | UNTREATED | | BNF 24 HR PRIOR | | BNF 48 HR PRIOR | |
|---|---|---|---|---|---|---|
| METABOLITE | TYPE II | CLARA | TYPE II | CLARA | TYPE II | CLARA |
| Origin | N.D.* | 4.9 | 4.9 | 4.4 | 4.7 | 11.6 |
| 9,10-Diol | 63.1 | 72.4 | 69.0 | 61.3 | 55.1 | 65.2 |
| 7,8-Diol | 18.9 | 19.6 | 25.0 | 24.9 | 20.8 | 11.6 |
| 4,5-Diol | 1.9 | 1.3 | 2.0 | 2.8 | 2.5 | 2.8 |
| Unknown | 3.5 | 0.8 | N.D. | 1.2 | 1.9 | 1.5 |
| Monohydroxys | 4.0 | 1.5 | 4.0 | 4.9 | 6.3 | 3.9 |
| Quinones | 3.7 | N.D. | N.D. | N.D. | 8.6 | 4.4 |
| BaP | 93.8 | 78.1 | 98.5 | 79.5 | 91.1 | 71.7 |

N.D. = Not Detected.

Binding of $^{3}$H-BaP to DNA of Lung Cell Cultures.

The radioactivity associated with the DNA of cells from the cultures described above is shown in Table 7. The levels of binding were proportional to the metabolism of $^{3}$H-BaP as indicated by the TLC analysis of metabolites in the medium. In most cases, the highest binding was seen in DNA from cultures enriched in Clara cells; however, pretreatment with BNF which increased the metabolism of $^{3}$H-BaP by type II cells also

increased the binding to DNA in these cells. The data in Table 7 indicate some amount of association of radioactivity with the cellular DNA in the absence of metabolic activity toward $^{3}$H-BaP, e.g., in the case of the cells from untreated rats. This level corresponds to about 1 x $10^{-3}$ pmole $^{3}$H-BaP/microgram DNA.

**TABLE 7**

Binding of $^{3}$H-BaP to DNA in Primary Rat, Mouse and Hamster Lung Cell Cultures

The values are the specific radioactivity of the DNA in the CsCl gradient fraction having the highest absorbance at 260 nm. Blank values have been subtracted.

| SPECIES/TREATMENT | CELL TYPES | PMOLES/MICROGRAM DNA |
|---|---|---|
| Rat/Untreated | Type II | 0.90 x $10^{-3}$ |
| Rat/Untreated | Clara | 0.62 x $10^{-3}$ |
| Rat/BNF 24 hr Prior | Type II | 0.37 x $10^{-3}$ |
| Rat/BNF 24 hr Prior | Clara | 0.97 x $10^{-3}$ |
| Rat/BNF 48 hr Prior | Type II | 2.67 x $10^{-3}$ |
| Rat/BNF 48 hr Prior | Clara | 2.43 x $10^{-3}$ |
| Mouse/Untreated | Type II | 0.10 x $10^{-3}$ |
| Mouse/Untreated | Clara | 2.54 x $10^{-3}$ |
| Mouse/BNF 24 hr Prior | Type II | 1.10 x $10^{-3}$ |
| Mouse/BNF 24 hr Prior | Clara | 6.30 x $10^{-3}$ |
| Mouse/BNF 48 hr Prior | Type II | 1.93 x $10^{-3}$ |
| Mouse/BNF 48 hr Prior | Clara | 3.52 x $10^{-3}$ |
| Hamster/Untreated | Type II | 0.45 x $10^{-3}$ |
| Hamster/Untreated | Clara | 1.40 x $10^{-3}$ |
| Hamster/BNF 24 hr Prior | Type II | 0.45 x $10^{-3}$ |
| Hamster/BNF 24 hr Prior | Clara | 1.51 x $10^{-3}$ |
| Hamster/BNF 48 hr Prior | Type II | 1.44 x $10^{-3}$ |
| Hamster/BNF 48 hr prior | Clara | 2.26 x $10^{-3}$ |

$O^{6}$-EtG Concentrations in Hamster Tracheal DNA after Various Doses of DEN.

Male hamsters were given DEN at doses of 50, 100 or 200 mg/kg i.p., and killed 12 hr later. Tracheas were removed and frozen at -80°C until DNA extraction and analysis. To collect the cells, the tracheas were cut longitudinally and the epithelium scraped into phosphate buffer. DNA was isolated by the method of Leadon and Cerutti (1982) and alkylated guanine

analyzed as decribed. Cells from 10 tracheas were used for each analysis. The results shown in Figure 1 indicate a clear dose-response relationship for the formation of $O^6$-EtG in tracheal DNA, consistent with the hypothesis that the formation of this alkylated base is related to tumorigenesis in this tissue.

Persistence of $O^6$-EtG in Hamster Tracheal DNA after a Single Large Dose of DEN.

Hamsters were given DEN at a dose of 200 mg/kg i.p.and sample groups of 10 animals killed at 3, 12, 24, and 48 hr posttreatment. $O^6$-EtG content of tracheal DNA was measured as above, and the results are shown in Figure 2. The maximum level of $O^6$-EtG was found at 12 hr posttreatment, and the absolute value was in good agreement with the results found previously.

$O^6$-EtG Formation in Hamster Tracheal DNA after Subchronic DEN Exposure.

Hamsters were treated twice weekly for 4 weeks with DEN (20 mg/kg, s.c.), and 24 hr after the last treatment they were given a challenge dose of 200 mg DEN/kg i.p. At 3, 12, 24 and 48 hr after this challenge, sample groups of 10 hamsters were killed and the $O^6$-EtG content of tracheal DNA measured as described. Figure 2 shows the results of this experiment. The pretreatment with DEN did not alter the formation and removal of $O^6$-EtG in tracheal DNA. This result suggests that neither the enzymes involved in DNA metabolism nor those concerned with repair of $O^6$-EtG in DNA were affected by the pretreatment.

Accumulation of $O^6$-EtG in Hamster Tracheal DNA During Subchronic Exposure to DEN.

Hamsters were treated twice weekly for 8 weeks with DEN (20 mg/kg, s.c.) and sample groups of 10 animals killed at 3 and 24 hr after the last treatment in weeks 1, 4, and 8. $O^6$-EtG content of tracheal DNA was measured as above. The results shown in Figure 3 indicate an accumulation of $O^6$-EtG during the treatment period, suggesting that the rate of repair of this alkylated base in tracheal DNA is substantially slower than the rate of formation at the given dose of DEN.

Cell Specificity in Hamster and Rat Pulmonary DNA Akylation after DEN or Ethyl Methanesulfonate (EMS) Treatment.

After an acute dose of either 100 or 200 mg DEN/kg, i.p., the DNA in isolated populations of hamster pulmonary cells was found to contain $O^6$-EtG as shown in Figure 4. Cell preparations enriched in either type II or Clara cells were analysed as in the case of the tracheal cells. The concentration of $O^6$-EtG was consistently and significantly higher in the DNA of Clara cell-enriched populations than in the DNA of alveolar type II or macrophage-enriched populations. The variation in $O^6$-EtG content between different Clara cell preparations may be related to the

degree of enrichment of the population with Clara cells. For example, the values obtained for $O^6$-EtG content with Clara cells from hamsters treated with 100 mg DEN/kg were expt. 1: 896 umol/mol G and expt. 2: 454 umol/mol G. The degree of enrichment in Clara cells was 56% and 41%, respectively. In the case of populations enriched in type II cells, higher concentrations of $O^6$-EtG were not found associated with increased purity of the population in type II cells. Cross contamination of type II populations with Clara cells, and vice-versa, was always less than 1%.

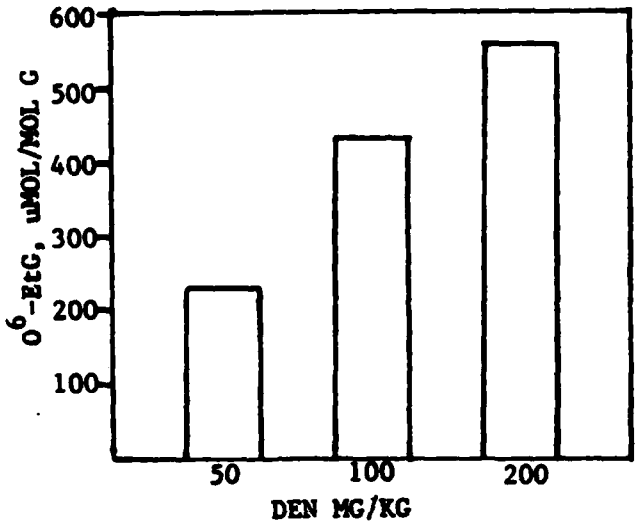


Figure 1. Formation of $O^6$-EtG In Hamster Tracheal DNA after Various Doses of DEN.

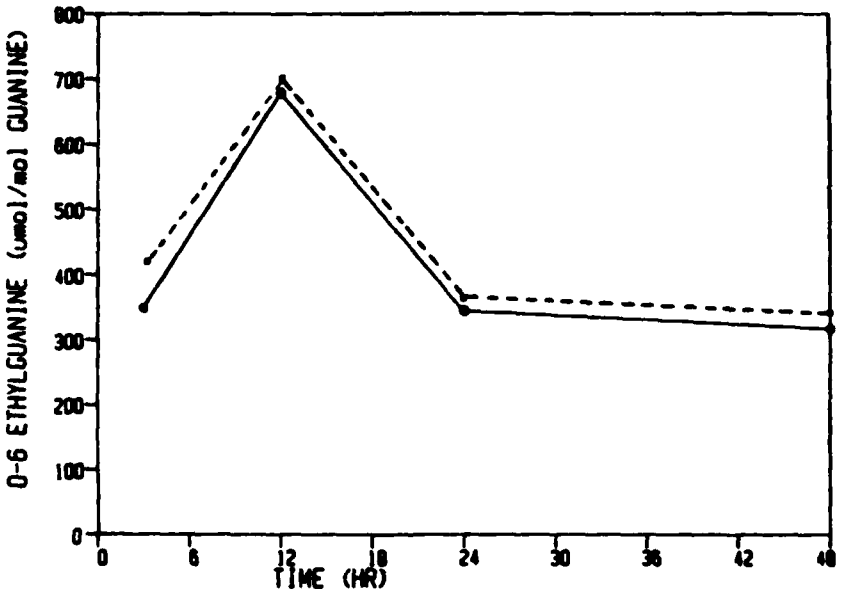


Figure 2. $O^6$-EtG Content of Hamster Tracheal DNA after a Single Large Dose of DEN. Solid line: pretreated with DEN as described in the text; Broken line: not pretreated.

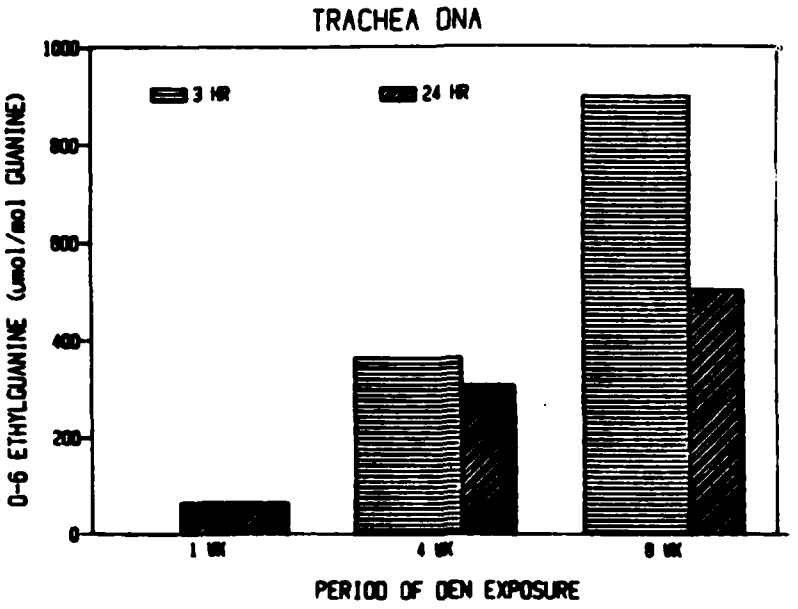


Figure 3. $O^6$-EtG Content of Hamster Tracheal DNA During Subchronic Exposure to DEN.

Average concentrations of $O^6$-EtG in DNA of Clara cell-enriched fractions isolated from duplicate groups of hamsters given 200 mg DEN/kg were less than those found after the lower dose of 100 mg/kg (e.g., at 12 hr, 381 vs. 675 umol/mol G; at 24 hr, 148 vs. 329 umol/mol G). This may be attributable to direct toxic effects of the DEN on the cells at the higher dose. Similar to the results found with tracheal DNA, the maximum level of $O^6$-EtG was found at 12 hr posttreatment, declining by about 1/2 by 24 hr at both dose levels. Estimates of the apparent half lives of $O^6$-EtG in Clara cell DNA were derived from semi-logarithmic plots of the data, and were 11-12 hr for the lower dose and 8-9 hr for the higher dose. Because of the low and variable level of alkylation in the DNA of type II cells and macrophages, quantitation of the persistence of $O^6$-EtG in these cells was not possible. Trace levels of $O^6$-EtG were detected in cell types present in other elutriator fractions but they were much lower than those in either type II or Clara cell-enriched populations. These results suggest that, in the hamster, Clara and type II alveolar cells represent the major target cells for DEN alkylation of DNA.

$O^6$-EtG in DNA of Rat Pulmonary Cells.

The distinctive pattern of $O^6$-EtG levels seen among the various cells in the hamster was not repeated in the rat. At 18 hr posttreatment with 200 mg DEN/kg i.p., the $O^6$-EtG content was similar for DNA of Clara cell-enriched (23.5% Clara cells) and alveolar type II cell enriched (51.7% type II) populations, with levels of 68.9 and 60.2 umol/mol G, respectively. These values

indicate that the type II cell DNA may be similarly alkylated in the 2 species, but the Clara cell DNA in the rat is much less susceptible to akylation by DEN. Rat pulmonary cells in other elutriator fractions showed either undetectable or only trace levels of $O^6$-EtG in their DNA.

Important differences exist between the hamster and rat in the location of Clara cells. In the hamster, Clara cells participate in lining the lobar, segmental, and subsegmental bronchi, and respiratory and terminal bronchioles, whereas in the rat, Clara cells are restricted to the more peripheral airways and respiratory and terminal bronchioles (Reznik-Schuller and Reznik, 1979). The composition of the tracheal epithelium is also characteristically unlike in the 2 species. Clara cells account for 41% of the cell population in the hamster, but are not identifiable in the rat trachea. The smooth endoplasmic reticulum (SER), the site of oxidative metabolism, is dispersed throughout the cytoplasm of hamster Clara cells but is restricted to the apical region in rat Clara cells. The wider distribution of Clara cells in the hamster respiratory tract relative to the rat, and greater abundance of SER in the hamster cells may contribute to the higher levels of $O^6$-EtG found in DNA of hamster whole lungs and in Clara cell-enriched populations.

$O^6$-EtG in Pulmonary Cells During Subchronic Exposure to DEN.

In contrast to the results reported above for tracheal cells, no $O^6$-EtG was detected in isolated hamster pulmonary cells until the 4th week of treatment with DEN (20 mg/kg s.c., twice weekly). At that time $O^6$-EtG was found in Clara cell DNA (260 umol/mol G), type II cell DNA (150 umol/mol G) and also in macrophage DNA (160 umol/mol G). The latter finding has been inconsistent (cf. Figure 4), and may not be due to alkylation of macrophage DNA directly. Possible explanations are that the subchronic treatment with DEN caused significant cell death in the lung, and the lavage procedure used to obtain macrophages also collected sloughed epithelial cells. Macrophages may also have scavenged dead cells that contained alkylated DNA. Therefore, the conclusion that macrophages in fact metabolized DEN to its alkylating intermediate must await further studies.

Metabolism of DEN by Hamster Pulmonary Cells and Hepatocytes in Culture.

In short- term culture freshly isolated populations of pulmonary cells and hepatocytes metabolized DEN to a reactive intermediate which alkylated cellular DNA (Figure 5). The concentration of $O^6$-EtG was much higher in hepatocyte DNA ($.464 \times 10^{-12}$ mol/$10^6$ cells) than in pulmonary cells. Among the

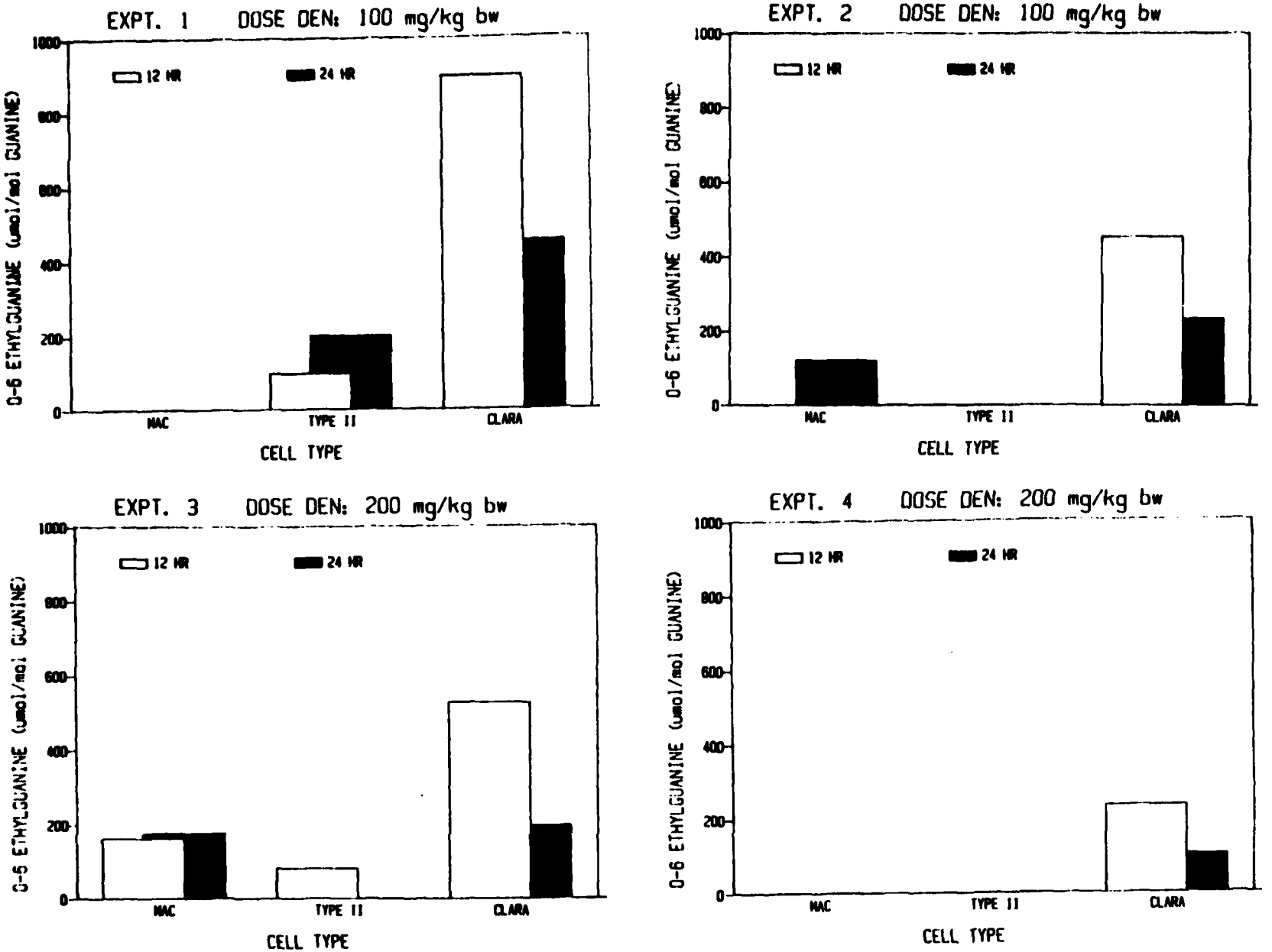


Figure 4. $O^6$-EtG Content of Hamster Lung Cells After In Vivo Exposure to DEN.

pulmonary cells, Clara cells showed the highest level of alkylation ($0.114 \times 10^{-12}$ mol/$10^6$ cells, with less in the type II cultures ($0.072 \times 10^{-12}$ mol/$10^6$ cells) and least in the macrophages ($0.040 \times 10^{-12}$ mol/$10^6$ cells). Cross contamination between type II and Clara cell populations was less than 1%. Cultures prepared with cells in the other elutriator fractions showed much lower levels of $O^6$-EtG, which appeared to be related to their content of Clara cells. In general, the relative levels of $O^6$-EtG found among the cells exposed to DEN in culture corresponded to the amounts found after in vivo exposure to DEN, as described above.

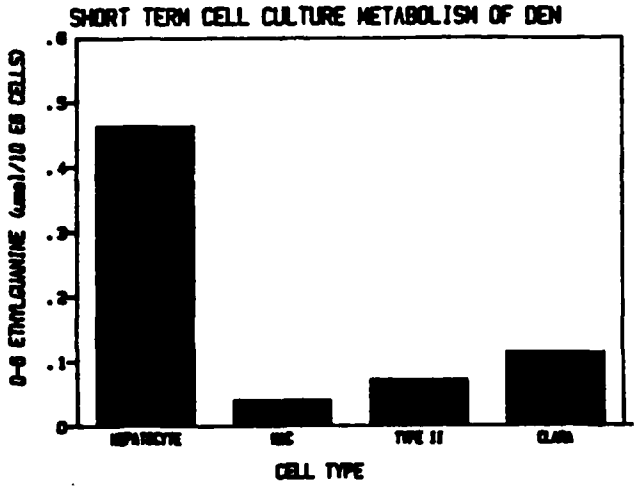


Figure 5. $O^6$-EtG Content of Hamster Cells After In Vitro Exposure to DEN.

The results of these studies are consistent with the hypothesis that $O^6$-EtG may be a critical lesion in the initiation in hamster respiratory tract carcinogenesis. The experiments with isolated cells indicate that metabolism and DNA binding can occur entirely within the cells that are the targets for carcinogenesis. Further, those cell types experiencing neoplastic transformation following DEN treatment show quantitatively greater amounts of $O^6$-EtG in their DNA than those which do not. With subchronic repeated exposure, $O^6$-EtG was found to accumulate in cells of the tracheal epithelium, from which arise the malignant tumors. Since DEN and many other chemical carcinogens require metabolic processing in order to bind to DNA, and presumably initiate cancers as a result, it appears especially important to study the specific metabolic pathways in target and nontarget cells and tissues. The information obtained from experimental systems could then be compared with metabolic pathways in human tissues obtained at autopsy or as specimens at surgery, in order to aid in estimation of human risk from exposure to the chemicals in question.

## REFERENCES


Cohen, G. M., Haws, S. M., Moore, B. P. , and Bridges, J. W. (1976),Benzo(a)pyren-3-yl hydrogen sulphate, a major ethyl acetate extractable metabolite of benzo(a)pyrene in human, hamster and rat lung cultures. Biochem. Pharmacol. 25:2561-2570.

Leadon, S. A., and Cerutti, P. A., (1982), A rapid and mild procedure for the isolation of DNA from mammalian cells. Anal. Biochem. 120:282-288.

Rasmussen, R. E. and Fong, A. T., (1984), Cellular Biochemistry and Cytogenetics in a Rat Lung Tumor Model. AFAMRL-TR-84-059, Wright-Patterson AFB, Ohio.

Rasmussen, R. E., and Painter, R. B., (1966), Radiation-stimulated DNA synthesis in cultured mammalian cells. J. Cell Biol. 29:11-19.

Rasmussen, R. E., and Wang, I. Y., (1974), Specific metabolism of benzo(a)pyrene depends on the inducer of hydroxylase activity. Cancer Res. 34:2290-2295.

Reznick-Schuller, H., and Reznick, G., (1979), Experimental pulmonary carcinogenesis. Int. Rev. Exp. Pathol. 20:211-281.

Swann, R. F., and Magee, P. N., (1968), Nitrosamine-induced carcinogenesis. The alkylation of nucleic acids of the rat by N-methyl-N-nitrosourea, dimethylnitrosamine, dimethylsulfate, and methylmethanesulfonate. Biochem. J. 110:39-47.




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